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Transmembrane Partitioning of Boron and Other Elements in RAW 264.7 and HL60 Cell Cultures

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ABSTRACT

The trace element boron is essential for all higher plants and is beneficial or has been established as essential for several animal models of human nutrition. To help identify the biomolecules that require boron for function in humans, we determined whether intracellular boron is retained against a concentration gradient. Cells (Abelson leukemia virus BALB murine monocyte–macrophage RAW 264.7 [RAW] and HL60) and supplemented media (Dulbecco's modified essential media [+ 10% fetal calf serum] and Iscove's modified Dulbecco's medium [+ 5% fetal calf serum], respectively) were analyzed for mineral concentrations after culture and subculture. Special corrections were made for trapped extracellular media in cell pellets and endocytosed media. For RAW cells, the partitioning coefficients (PC; intracellular/extracellular ratios) were, in rank order, as follows: Mn, 110; Fe, 67; P, 65; Zn, 32; K, 15; Cu, 7.1; Mg, 4.3; B, 1.7; Ca, 0.4; Na, 0.3. For HL60 cells, the partitioning coefficients were, in rank order, as follows: Mn, 212; Zn, 211; P, 123; K, 21; Fe, 16; Mg, 11; B, 1.7; Ca, 0.8; Na, 0.3. Trapped extracellular media was estimated to be 6.7 ± 0.8%; trapped extracellular and endocytosed media together was 24.8 ± 0.3% of the mass within the isolated cell pellets. The partitioning coefficients indicate a positive gradient for intracellular accumulation of boron, zinc, phosphorus, manganese, magnesium, potassium, iron, and copper in RAW264.7 and HL60 cells. Specifically, the data indicate the existence of a selective boron-

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binding molecular species within the cell or the existence of a boron-specific membrane transporter.

Index Entries: Boron; membrane partitioning; RAW 264.7; HL60; partitioning coefficients; zinc; phosphorus; manganese; magnesium; potassium; iron; copper.

INTRODUCTION

The distribution of an essential element between the intracellular and extracellular compartments is determined by its binding affinities with biomolecules present in each compartment and the activities of ion transporters and channels in the membrane partition between these compartments *(1).* Certain trace elements such as zinc, copper, iron, and manganese are stabilized intracellularly *(2,3)* by covalent incorporation into biomolecules following uptake by specific mechanisms *(4).* On the other hand, some elements (i.e., potassium) are sequestered by selective ion transport mechanisms that concurrently externalize other elements (i.e., calcium and sodium) against significant concentration gradients *(5–7).* Thus, the intracellular distribution of a given element is dependent on its unique binding properties and specialized transport systems that result in either positive or negative concentration gradients *(1).*

The element boron is essential for all higher plants *(8,9)* and for at least some organisms in each of the other phylogenetic kingdoms Eubacteria *(10),* Stramenopila (brown algae and diatoms) *(11),* and Fungi *(12).* Physiologic concentrations of boron are needed to support metabolic processes in several species in Animalia *(13–18).* For example, embryological development in fish *(13)* and frogs *(14)* does not proceed normally in the absence of boron. There is evidence that chicks *(15),* rats *(16,17),* and pigs *(18)* require physiological amounts of boron to support normal biologic processes, including immune function and bone development. In humans, boron is under apparent homeostatic control *(19)* and is beneficial for immune function *(20,21).* Therefore, specific, but yet unidentified, cellular biomolecules must interact with boron.

Boron is an integral component of several biomolecules, where it is thermodynamically stabilized in a covalent bond *(22–25)* or a boroester *(26).* The presence of boron in these molecules is essential; in its absence, they no longer perform their normal physiologic functions. Boron association with molecules present in animal cells has been studied recently and novel binding species were recognized (i.e., *S*-adenosyl methionine) *(27).* Such binding interactions may influence boron partitioning across the cell membrane to create a positive concentration gradient, but this hypothesis has not been tested.

Analysis of equilibrium distributions of intracellular and extracellular boron concentrations could be used to study boron partitioning across the cell membrane. If intracellular boron is retained against a concentration

gradient, this might indicate the presence of intracellular boron-binding species or the existence of boron-specific transporters on the plasma membrane. To assess boron distributions in this way, large quantities of cells are needed to provide adequate cell mass for accurate elemental analysis of intracellular boron and enhanced precision of analytical parameters such as packed cell volumes.

In this study, we have examined the concentrations of intracellular and extracellular boron and compared them to those of other elements in cultured cells. We find that, similar to zinc, copper, iron, and manganese, higher concentrations of boron are maintained in the intracellular compartment against a gradient of lower extracellular concentrations.

METHODS AND MATERIALS

Abelson leukemia virus BALB murine monocyte–macrophage RAW 264.7 cells were obtained from American Type Culture Collection (Rockville, MD). In each experiment, RAW 264.7 cells were grown in Dulbecco's modified essential media (DMEM; Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS; Gibco) in either cell culture flasks (Corning Inc., Acton, MA) or Nunc (Nalgene, Rochester, NY) cell culture plates and subcultured before exceeding approx 80% confluence to prevent cellular differentiation. The adherent cells were scraped free and resuspended at 0.5×10^6/mL in 1.5–2.5 L of DMEM + 20 m*M* HEPES (Sigma Chemical Co., St. Louis, MO) in stir bottles (Corning Inc., Acton, MA). After 48 h in culture, the RAW 264.7 cell density had increased to $(2–2.5) \times 10^6$/mL. The suspension culture was distributed into 250-mL centrifuge bottles and centrifuged at 200*g* for 10 min. Samples of the supernatant media for elemental analysis were transferred into trace-metal-free 50-mL Teflon tubes (Daigger, Vernon Hills, IL). Cell pellets were resuspended in a smaller volume of the same media and combined in a preweighed trace-metal-free 50-mL Teflon tube and aliquots were taken for cell counting before centrifugation at 450*g*. Supernatant media was removed by aspiration and the pellet mass determined gravimetrically in preweighed trace-metal-free Teflon tubes.

Fresh media, as well as media at equilibrium with cell suspensions, and pelleted cell samples were identically treated along with analytical control samples by using redistilled 16 *M* nitric acid and 30% hydrogen peroxide (Ashland Chemical, Columbus, OH) as digestion reagents. Digested samples were analyzed by inductively coupled plasma–emission spectrophotemetry (ICP; Perkin-Elmer, Norwalk, CT) to determine elemental concentrations.

For comparison, a parallel study of the distribution of boron and other cellular elements was conducted on HL60 cells (American Type Culture Collection, Rockville, MD). These cells were grown in Iscove's modified Dulbecco's medium (IMDM) + 5% FCS (Sigma–Aldrich Co., St. Louis,

MO) in multiple 175-cm^2 cell culture flasks (Corning, Acton, MA) and otherwise prepared for analysis identically to the RAW 264.7 cells.

For measuring trapped extracellular media in cell pellets, 350 nmol of bromine was added per milliliter of cells suspended in media, mixed, and incubated briefly (10 min) before centrifugation at 450*g* for 10 min. Bromine does not bind to either cell or endocytic membranes and has a low cell penetrance. The supernatant media were removed and the cell pellets were resuspended in fresh HEPES-buffered saline. External media that had been trapped in the cell pellets were diluted in the saline supernates when the cell suspensions were centrifuged at 450*g* for 10 min. Aliquots of the supernatant saline were removed for bromine analysis by high-performance liquid chromatography (HPLC). The amount of bromine marker present in the saline suspension was compared to the amount of bromine present in the original media to determine the amount of media that had been present in the original cell pellet.

In a separate set of samples, the amount of extracellular and endocytosed media present in cell pellet samples was determined by incubating cell cultures overnight to allow the fluorescent marker Alexafluor (Molecular Probes, Eugene, OR) to attain equilibrium distribution. Alexafluor also does not bind to either cell or endocytic membranes and does not penetrate the cell. The following morning, the cell suspensions were centrifuged at 450*g* for 10 min to pellet the cells and the Alexafluor fluorescence present in the cell pellets were quantitated by fluorescence spectrophotometry. The amounts of minerals in extracellular and endocytosed media present in the cell pellets were subtracted from the total amount of each element in the cell sample and these contributions were mathematically corrected to determine the elemental concentrations within the cells. The concentrations of the elements within the cells were compared with their concentrations in the media and the ratio reported on a per-milliliter basis as the partitioning coefficients. Elemental concentrations in the cells and media were compared using a one-tailed *t*-test.

RESULTS

Through application of large-scale cell culture methods, we obtained yields of billions of cells per sample. The number of cells in the sample sets were in the range approx (1–3) $\times 10^9$ cells per sample and provided high accuracy in our mass and volume determinations. For RAW 264.7 cells, the intracellular concentrations of the measured elements varied with the rank order K (93 m*M*) > P > Na >> Mg >> Ca > Fe > Zn >> Cu > Mn > B (*see* Table 1). The extracellular concentrations varied in the rank order Na (161 m*M*) >> K >> Ca > Mg > P >> Zn > Fe > Cu > B >> Mn. The intracellular/extracellular ratios indicated in Table 1 show the partitioning coefficients for each element. Concentrations of calcium and sodium were

Table 1
Elemental Concentrations in RAW 264.7 Cells and Media at Equilibrium Distribution[1]

Sample	Element									
	B μM	Mn μM	Fe μM	P μM	Zn μM	K μM	Cu μM	Mg μM	Ca μM	Na μM
n	4	4	4	4	4	4	4	4	4	4
Cells	2.32 ± 0.37[2]	9.24 ± 0.75	317 ± 32	66000 ± 7000	207 ± 26	93400 ± 14700	15.5 ± 2.6	6770 ± 680	821 ± 67	47900 ± 560
Media	1.35 ± 0.17	0.12 ± 0.03	4.7 ± 0.3	1020 ± 20	8 ± 2	6380 ± 40	3.15 ± 1.48	1570 ± 0	1970 ± 20	161000 ± 30(
C/M ratio	1.7	110	67	65	32	15	7.1	4.3	0.4	0.3
P value	0.03	0.0006	0.001	0.001	0.002	0.005	0.0008	0.003	0.0002	0.001

[1] Cells were grown in DME supplemented with 10% FCS in suspension at concentrations up to 2×10^6/mL in stir bottles. Cell suspensions were centrifuged at 450*g* for 10 min, and then immediately resuspended in smaller volumes of the same media, combined, and counted in gravimetrically determined volumes of media in trace-element-free Teflon tubes. Suspensions were centrifuged at 450*g* for 10 min and the supernatant media removed. Cell pellet mass and samples of media were determined gravimetrically, subtracting the preweighed tube weight from the total. Cell and media samples were digested and then analyzed by ICP. Experiments were reproduced in *n* independent analytical determinations.

[2] Elemental concentrations in each cell and media sample were calculated by determining the mass of each element per unit volume of sample. Values shown are the means ± SEM.

markedly lower in the intracellular compartment, and the concentrations of all other trace elements studied were higher intracellularly than extracellularly. The rank order of partitioning coefficients observed was Mn > Fe ~ P > Zn > K > Cu > Mg > B > Ca > Na.

For HL60 cells, intracellular concentrations of the measured elements varied in the rank order K (96 m*M*) ~ P > Na >> Mg >> Ca >> Zn > Fe >> Cu > Mn > B (*see* Table 2). The extracellular concentrations varied in the rank order Na (120 m*M*) >> K > Ca > P > Mg >> Fe >> Zn > B >> Cu > Mn. As in the RAW 264.7 cells, concentrations of calcium and sodium in HL60 cells were lower in the intracellular compartment than they were extracellularly and the concentrations of all other trace elements measured were higher intracellularly than extracellularly. In HL60 cells, however, the highest intracellular partitioning coefficients were noted for zinc and manganese, where the cells had sequestered nearly all of these elements from the media. The rank order of partitioning coefficients observed was Mn ~ Zn > P > K > Fe ~ Mg > B > Ca > Na.

Bromine analysis indicated that the trapped extracellular media surrounding the pelleted cells comprised 6.7 ± 0.8% of the pellet volume. The distribution of Alexafluor at equilibrium suggested that the trapped extracellular and endocytosed media present within the cells comprise 24.8 ± 0.3% of the mass within the isolated cell pellets.

DISCUSSION

Intracellular Boron Sequestration

The higher intracellular boron concentrations observed in association with the cultured cells seems likely to be the result of selective boron binding by molecular species within the cell. Candidate species include NADH and NAD^+, previously recognized as having the highest known affinity for boron among molecules present in animal cells *(28),* because the *cis*-hydroxyl moieties present on their ribose pairs form relatively stable molecular complexes with boron. Recently, the diadenosine polyphosphate (A_2P_n) species were found to have still greater boron-binding affinities *(27).* The two riboses of A_2P_n separated by *n* phosphate residues (*n* = 2–6) become increasing capable of cooperatively binding boron as the number of intervening phosphates is increased. With intramolecular separation, the riboses become increasingly capable of forming stable unimolecular borospirane configurations. Similarly, glycosylated lipids and proteins with multiple neighboring mannose and sialic acids residues appear equally capable of forming cooperative boron-binding sites. Furthermore, boron binding with the *cis*-diol of ribose of such species becomes magnified when local charge distributions contribute to the stability of the association. This is exemplified by *S*-adenosylmethionine, whose affinity for boron is greater than all other molecular species tested *(27).*

Table 2
Elemental Concentrations in HL60 Cells and Media at Equilibrium Distribution[1]

Sample	Element									
	B µM	Mn µM	Zn µM	P µM	K µM	Fe µM	Cu µM	Mg µM	Ca µM	Na µM
n	4	4	4	4	4	4	4	4	4	4
Cells	2.38 ± 0.62[2]	5.70 ± 0.92	278 ± 4	96200 ± 1900	96700 ± 1700	108 ± 7	16.1 ± 2.9	8020 ± 230	1320 ± 50	38500 ± 290
Media	1.19 ± 0.19	0.003 ± 0.001	1.4 ± 0.1	781 ± 18	4590 ± 140	8.9 ± 1.9	0.1 ± 0.1	702 ± 10	1570 ± 30	120000 ± 50(
C/M ratio	1.7	212	211	123	21	16	9.2	11	0.8	0.3
P value	0.01	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	<0.0001	0.0008	<0.0001

[1] Cells were grown in IMDM supplemented with 5% donor calf serum in suspension at concentrations up to 2×10^6/mL in 175-cm^2 flasks. Cell suspensions were centrifuged at 450*g* for 10 min and then immediately resuspended in smaller volumes of the same media, combined, and counted in gravimetrically determined volumes of media in trace-element-free Teflon tubes. Suspensions were centrifuged at 450*g* for 10 min and the supernatant media removed. Cell pellet mass and samples of media were determined gravimetrically, subtracting the preweighed tube weight from the total. Cell and media samples were digested and then analyzed by ICP. Experiments were reproduced in *n* independent analytical determinations.

[2] Elemental concentrations in each cell and media sample were calculated by determining the mass of each element per unit volume of sample. Values shown are the means ± SEM.

[3] In some cases, elemental concentrations in media were below analytical detection limits. Cell: media ratios shown for Mn and Cu are the means of cases where media concentrations could be determined.

We chose to perform our studies in a cell culture model because in animal tissues, the heterogeneity of cell types present and the potential differences in the boron utilization by the cell types present would make it difficult to delineate actual concentration gradients. The uptake, retention, and distribution would be far more uniform in a culture model such as we applied. However, even in animal models where homogeneity of cell types cannot be assured, there are obvious concentration gradients apparent between plasma and organ boron concentrations. In a recent study that measured boron concentrations in organ and tissues collected from rats *(29),* the control animal plasma was 1.94 ± 0.17, whereas tissues such as the brain (0.76 ± 0.02) and liver (0.66 ± 0.10) maintained far lower boron concentrations. On the other hand, the concentration of boron in muscle (3.69 ± 0.54) and large intestine (3.08 ± 0.17) was far higher than that in blood.

Intracellular boron sequestration could also be the result of activities of a transporter that maintains a boron gradient across the plasma membrane. Specifically, the higher intracellular boron concentrations may indicate the existence of a boron-specific membrane transporter. There are several lines of emerging evidence for existence of such a transporter. For example, in a metabolic study with postmenopausal women, gastrointestinal absorption of inorganic boron and subsequent urinary excretion *(19)* was near 100%. However, a 9.0-fold increase in dietary boron (0.36 mg to 3.0 mg B/d; an increase from the 5th to 95th percentile of usual boron intake) during the study yielded only a 1.5-fold increase in plasma boron concentrations (5.92 ± 4.16 μg to 8.79 ± 5.18 μmol B/L). The relatively small change in blood boron values coupled with large increases in urinary boron values indicates a strong boron gradient across the plasma membrane in the kidney. In female rats, supplementation with high amounts of boron (9.25 mmol/L water) for 21 d, caused an increase in plasma boron concentrations, but an undefined homeostatic mechanism concurrently eliminated any excess of boron from the liver and brain against their own concentration gradients *(30).* In cows, the percent of filtered boron reabsorbed by the kidneys decreased significantly with increased boron intake *(31).* The percentage of filtered boron resorbed by the kidneys decreased significantly with increased boron intake *(31),* a finding that suggests physiologic regulation through membrane transport.

Novel Correction for "Trapped" Media

In our samples, approx 6% of the apparent cell pellet mass was actually extracellular media "trapped" between and above the cells and an additional 18% of the apparent cell mass was media enclosed in pinocytic vesicles. The total amount of trapped media (approx 24%) was significantly less than that in leukocyte pellets (43%), as reported by Baron and Ahmed *(32).* In the earlier study, the meniscus of media over much smaller pellets probably contributed to the greater amount of extracellular media

present in their samples. In our study, a larger sample volume undoubtedly enhanced the analytical precision of our measurements. Failure to accurately assess and correct for media that was internalized within endocytic vesicles would result in an overestimation of intracellular concentrations of calcium, sodium, and any other components that are preferentially distributed outside the cells. It would also result in an underestimation of actual intracellular concentrations of elements sequestered by the cells (*see* Tables 1 and 2).

The media surrounding the cells and temporarily internalized in cellular endosomes was quantitated by media markers that do not exhibit nonspecific binding to membrane components or cross the plasma membrane to enter the cytosol. These are important considerations and not easily achieved. For example, the use of phenol red, the pH indicator present in many media formulations, as the marker for extracellular media trapped in the pellet is not appropriate. Phenol red exhibits significant nonspecific binding to cell membranes that results in significant overestimations of media trapped in cell pellets.

Through the application of Alexafluor as a media marker, we were able to quantify directly the amount of marker present in the cell pellets and determine the amount of media present in both the intracellular and extracellular compartments. Bromine has been used to measure the volume of the extracellular compartment in human studies, but to our knowledge, it has not previously been used to assess extracellular volumes in studies at the cellular level.

Our data indicate that RAW264.7 and HL60 cells selectively accumulate boron, copper, iron, magnesium, manganese, phosphorus, potassium, and zinc from the media. The partitioning coefficients for boron indicate a positive gradient for intracellular boron accumulation. Further research is needed to determine whether the phenomenon is caused by boron binding to high-affinity biomolecules or if selective boron transport mechanisms are responsible.

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